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### Heteropolymer-mediated clearance of immune complexes via erythrocyte CR1: mechanisms and applications.

**Lindorfer MA, Hahn CS, Foley PL, Taylor RP.**

Department of Biochemistry and Molecular Genetics, University of Virginia Health System, Charlottesville, VA, USA.

Opsonization of particulate pathogens by antibodies and complement can lead to their binding to the complement receptor (CR1), specific for C3b, on primate erythrocytes (E). This process of immune adherence may play a role in immunologic defense by immobilizing bacteria and viruses, thus preventing them from leaving the bloodstream to invade susceptible tissue and organs. Immune adherence of C3b-opsonized and immune complexed pathogens to E may also facilitate their transfer to, and destruction by, fixed tissue macrophages. We have used mAbs specific for CR1 crosslinked with pathogen specific mAbs to generate heteropolymers (HP) which can bind a wide range of substrates to primate erythrocytes. Both prototype and bonafide pathogens bound to primate E via HP are handled in the circulation of non-human primates in a manner which appears to be virtually identical to the mechanism by which C3b-opsonized substrates bound to E CR1 are cleared. In this process of focused phagocytosis, Fc receptors on the phagocytic cell engage the E-bound complex, CR1 is removed by proteolysis, and the entire immune complex and CR1 are internalized while sparing the E. It may be possible to use HP to target pathogens in the bloodstream in a wide range of therapeutic applications.

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### Producing bispecific and bifunctional antibodies.

**Das D, Suresh MR.**

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada.

Bispecific antibodies are artificially engineered monoclonal antibodies (MAbs) that consist of two distinct binding sites and are capable of binding two different antigens noncovalently. They can be produced by chemical cross-linkage, genetic engineering, or somatic hybridization. This chapter describes a rapid method using somatic fusion to generate hybrid hybridomas (quadromas). Two fluorescence-labeled hybridoma cell lines were fused with polyethylene glycol (PEG) to generate the quadroma. Generation of a quadroma secreting bsMAb against biotin and HRPO is described, along with a benzhydroxamic acid-agarose affinity chromatography procedure to purify the bsMAb-HRPO complex. This bsMAb can be used for ultrasensitive ELISA detection of biotinylated antigens. Essentially a similar method can be used for fusing any two hybridomas for therapeutic applications. Bifunctional antibodies are colinear molecules with one or more paratopes linked with diagnostic or therapeutic molecules. There are some limitations of therapeutic monoclonal antibodies in the clinic that can be overcome by engineering smaller and more effective antibody fragments. Here we describe a stepwise procedure for developing a bifunctional ScFv (bfScFv). We constructed a bfScFv from a hybridoma cell line using PCR strategies. The VL and VH gene segments are linked with a 45-bp linker and fused with a biotin mimic sequence at the 3' end. This engineered bifunctional antibody fragment gene could be expressed and the protein purified on a large scale in *Escherichia coli* as inclusion bodies. Such bifunctional antibody molecules could have useful applications in the area of immunodiagnosics and immunotherapy. Similar strategies can be used to incorporate a second single-chain antibody or any nonantibody entity such as a cytokine for therapeutic applications.

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**Primate erythrocyte (E) complement receptor (CR1) as an anchor site for bispecific-based therapies to clear pathogens or autoantibodies safely from the circulation.**

**Taylor RP, Ferguson PJ.**

Department of Biochemistry, University of Virginia School of Medicine, Charlottesville 22908, USA.

We have prepared cross-linked, bispecific complexes [heteropolymers (HP) and antigen-based heteropolymers (AHP)] that facilitate complement-independent binding of target model pathogens or autoantibodies to primate erythrocytes (E) via complement receptors (CR1). The method is based on using monoclonal antibodies (mAb) specific for CR1 that either are cross-linked to an mAb specific for a prototype pathogen (e.g., IgE) or are cross-linked to an autoantigen (e.g., dsDNA) that is recognized by circulating pathogenic autoantibodies in the autoimmune disease systemic lupus erythematosus (SLE). The underlying assumption in this research is that complexed ligands containing IgG bound to primate E CR1 should be recognized and processed via the same mechanism by which complement-opsonized immune complexes bound to E CR1 are cleared from the circulation and phagocytosed in the liver and spleen. Our work in experimental monkey models has demonstrated that binding of substrates to primate E via this method does indeed lead to the safe and rapid clearance of the target pathogens or autoantibodies from the circulation, without any lysis or loss of the E. Although a number of questions must still be resolved, it may be possible to generalize these findings and use this CR1-based approach to develop a simple noninvasive bispecific therapy that can be used to clear pathogens or autoantibodies from the circulation.

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- ▶ Antigens pre-bound to the primate erythrocyte complement receptor via cross-linked bispecific monoclonal antibody heteropolymers are rapidly cleared from the circulation. [J Immunol. 1993]
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### Abstract:

Bispecific antibodies are artificially engineered monoclonal antibodies (MAbs) that are capable of binding two different antigens noncovalently. They can be produced by engineering, or somatic hybridization. This chapter describes a rapid method using hybridomas (quadromas). Two fluorescence-labeled hybridoma cell lines were fused to generate the quadroma. Generation of a quadroma secreting bsMAb against biotin benzhydroxamic acid-agarose affinity chromatography procedure to purify the bsMAb used for ultrasensitive ELISA detection of biotinylated antigens. Essentially a similar method can be used to generate two hybridomas for therapeutic applications.

Bifunctional antibodies are colinear molecules with one or more paratopes for antigen binding. There are some limitations of therapeutic monoclonal antibodies in the form of engineering smaller and more effective antibody fragments. Here we describe a bifunctional ScFv (bfScFv). We constructed a bfScFv from a hybridoma cell line using segments are linked with a 45-bp linker and fused with a biotin mimic sequence. The bifunctional antibody fragment gene could be expressed and the protein purified by inclusion bodies. Such bifunctional antibody molecules could have useful applications in immunotherapy. Similar strategies can be used to incorporate a second single entity such as a cytokine for therapeutic applications.

### Keywords:

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### Design and expression of a stable bispecific scFv dimer with affinity for both glycophorin and N9 neuraminidase.

**Atwell JL, Pearce LA, Lah M, Gruen LC, Kortt AA, Hudson PJ.**

CSIRO, Division of Biomolecular Engineering, CRC for Diagnostic Technologies, Parkville, Victoria, Australia.

We have designed and produced a stable bispecific scFv dimer (bisFv) by non-covalent association of two hybrid VH-VL pairs derived from an anti-neuraminidase antibody (NC10) and an anti-glycophorin antibody (1C3). The bisFv dimer was demonstrated to have binding activity to the two respective target antigens and was evaluated as a reagent for rapid whole blood agglutination assays. The bisFv was expressed in the periplasm of *Escherichia coli*, from a secretion vector which comprised two cistrons in tandem under the control of a single lac promoter, inducible with IPTG. Each cistron encoded one of the hybrid VH-VL pairs, with V domains separated by a linker region encoding the five amino acids, Gly4Ser. The short linker region was designed to prevent association of VH and VL regions of the same molecule and favour the formation of dimers. The protein synthesized from each hybrid scFv cistron was directed to the *E. coli* periplasm by the inclusion of distinctive signal secretion sequences preceding each hybrid gene; from pel B of *Erwinia cartovora* and from gene III of fd phage. The bisFv was affinity-purified from culture supernatants via the C-terminal tag epitope FLAG and was shown, by FPLC on a Superose 6 column, to be consistent in size with that of a scFv dimer. The bisFv was stable for more than 4 months at 4 degrees C and was shown by BIAcore analysis to bind to either target antigen, human glycophorin, or tern N9 neuraminidase. Simultaneous binding to both target antigens was demonstrated when a pre-formed bisFv-neuraminidase complex was shown to bind to immobilized glycophorin. In whole blood agglutination assays, the bisFv dimer was able to agglutinate red blood cells when crosslinked with an anti-idiotypic antibody (3-2G12) binding to the NC10 combining site, but no agglutination occurred on binding the antigen neuraminidase. These results are a function of the topology of the epitopes on neuraminidase and have implications for the use of relatively rigid bifunctional molecules (as bisFv dimers) to cross link two large

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- ▶ Effect of domain order on the activity of bacterially produced bispecific single-chain Fv antibody. [Mol Biol. 2003]
- ▶ scFv multimers of the anti-neuraminidase antibody NC10: length of the linker between VH and VL domains dictates precisely the transition between diabodies and triabodies. [Protein Eng. 1999]
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**Single-chain antibody streptavidin fusions: tetrameric bifunctional scFv-complexes with biotin binding activity and enhanced affinity to antigen.**

**Kipriyanov SM, Breitling F, Little M, Dübel S.**

German Cancer Research Center, Recombinant Antibody Research Unit, Heidelberg, Germany.

To increase the avidity of single-chain antibodies (scFv) for their antigen, we have fused them to core-streptavidin. The chimeric protein, expressed by the vector pSTE (plasmid for streptavidin-tagged expression) from Escherichia coli, can form tetrameric complexes, binds its antigen and contains four biotin binding sites per tetrameric complex. An additional cysteine inserted near the carboxy terminus further stabilised the complex. The scFv fusion protein tetramers could be enriched by affinity chromatography using the biotin analog 2-iminobiotin from periplasmic inclusion bodies after refolding. We have also shown that the scFv fusion protein could be used for direct detection of its antigen in ELISA when stained with biotinylated horseradish peroxidase. The affinity of the scFv-antibody complex was substantially increased by avidity effects due to the tetrameric structure. The biotin binding sites may be used for coupling other antibodies and molecules to form bispecific and bifunctional reagents.

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